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(71) Applicant: THOMAS JEFFERSON UNIVERSITY [US/US]; 11th and Walnut Streets, Philadelphia, PA 19107 (US).

(72) Inventors: ZALEWSKI, Andrew ; 619 Elkins Park, Elkins Park, PA 19117 (US). SHI, Yi ; 100 Old York Road, Apt. 504, Jenkintown, PA 19046 (US).

(74) Agents: JOHNSON, Philip, S. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, One Liberty Place, 46th Floor, Philadelphia, PA 19103-7301 (US).

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(54) Title: INTERFERON GENE THERAPY FOR THE TREATMENT OF VASCULAR DISORDERS

(57) Abstract

A method of modulating smooth muscle cell proliferation is provided whereby smooth muscle cells are transformed with genes coding for one or more interferon polypeptides. In addition, a method of inhibiting intravascular blockages is provided. Kits and compositions thereof are also provided.

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INTERFERON GENE THERAPY FOR THE TREATMENT OF VASCULAR DISORDERS

FIELD OF THE INVENTION

This invention is directed to interferon gene therapy. In particular, this invention is directed to interferon gene therapy for the treatment of vascular disorders.

BACKGROUND OF THE INVENTION

Coronary angioplasty results in successful nonsurgical revascularization in more than 90% of patients. More than 300,000 coronary angioplasty procedures were performed in the United States in 1990. However, the major limitation of coronary angioplasty is a 30-40% restenosis rate which occurs in the first six months following the procedure. The cellular basis for coronary restenosis includes intense smooth muscle cell (SMC) proliferation in response to vessel wall injury. This process is characterized by a change in SMC phenotype from the contractile to the sympathetic form, migration of both phenotypes from arterial media to the intima, subsequent synthesis and the secretion of extracellular collagenous matrix at the site of vessel wall injury. Clowes et al. *S.M. Circ. Res.* 56: 139-145 (1985); Austin et al., *J. Am. Coll. Cardiol.* 6: 369-375 (1985); Essed et al., *Br. Heart J.* 49: 393-396 (1983). Furthermore, it has become evident that the restenosis process is multifactorial, and relates to platelet and thrombin reactivity as well as several growth factors. Ip et al., *J. Am. Coll Cardiol.* 15: 1667-1687 (1990); Ross et al., *Cell* 46: 155-169 (1986); Gajdusek et al., *J. Cell Biol.* 85: 467-472 (1980). This multifactorial pathogenesis explains the

disappointing results of intensive clinical investigations over the past decade regarding treatments for restenosis.

Intensive experimental and clinical investigation for the prevention of restenosis has been conducted over the past decade. Several interventions, such as anti-platelet, anti-coagulation, anti-inflammatory and vasodilator therapies have shown favorable reduction in the severity of intimal proliferation following experimental balloon injury. Powell et al., *Science* 245: 186-188 (1989); Castellot et al., *J. Cell Physiol.* 124:21-38 (1985); Fox et al., *Science* 241: 453-456 (1988).

Recently, attempts have also been made to apply new mechanical devices to limit restenosis (e.g., stent, atherectomy, laser, rotablator, etc.). However, preliminary data showed a limited role of these interventions because while mechanical interventions improve the primary result of coronary angioplasty, the mechanical techniques extend vessel wall injury related to the procedure and are therefore unable to reduce SMC proliferation and the restenosis rate. Furthermore mechanical interventions can be applied only to a small group of patients with optimal coronary anatomy.

Application of antimitogenic therapy has also been suggested for prevention of restenosis. For example, concentrated heparin has been tested as an antiproliferative agent to control the problem of restenosis after angioplasty. Wolinsky and Thung, *JACC* 15(2): 475-481 (1990). γ-interferon has been identified as another potentially useful therapeutic for treatment of restenosis. WO 90/03189 issued April 5, 1990. However the dose of antiproliferative agents given by systemic administration is likely not high enough to achieve the desired effect. Therefore, agents which have been tested are not powerful enough to show a beneficial effect in more complex clinical situations.

The transfer of genes is a well-known phenomenon in biology and evolution of organisms. Gene therapy is a recently d veloped modality useful for introduction of a foreign gene into the target tissue in order to correct an inherited or

acquired disorder through the synthesis of designed gene products *in vivo*. For instance, endothelial cells may be genetically engineered with a gene for a heterologous protein useful as a therapeutic agent, such as soluble CD-4, Factor VIII, Factor IX, von Willebrand Factor, t-PA, urokinase, hirudin, interferons, tumor necrosis factor, interleukins, hematopoietic growth factor, antibodies, glucocerebrosidase, ADA, phenylalanine, hydroxylase, human growth hormone, insulin and erythropoietin. Such endothelial cells have been implanted in the vascular system of mammals via vascular grafts. WO 90/06997 issued June 28, 1990. Genetically engineered cells with a gene for a protein such as t-PA, urokinase, streptokinase, acidic fibroblast growth factor, basic fibroblast growth factor, tumor necrosis factor α, tumor necrosis factor β, transforming growth factor α, transforming growth factor β, atrial natriuretic factor, platelet-derived growth factor, endothelian, insulin, diphtheria toxin, pertussis toxin, cholera toxin, soluble CD4 and growth hormone may also be delivered to discrete blood vessel segments via double balloon catheterization methods. It has also been suggested that recombinant genes coding for proteins such as those provided above may be incorporated into vectors and introduced directly to cells on the walls of blood vessels *in vivo* using balloon catheter techniques. WO 90/ 11734 issued October 18, 1990. Such *in vivo* gene transfer has been demonstrated using a β-galactosidase marker gene expressed in a specific arterial segment *in vivo* by direct infection with a murine amphotropic retroviral vector or alternatively by lipid mediated transfection.

A method of modulating the proliferation of smooth muscle cells associated with restenosis in any vascular bed is greatly desired. Such method should be applicable to patients having a broad range of vascular disorders including coronary and peripheral stenoses (i.e., blockages). Further, the method should have high efficacy. Such methods, and kits and compositions thereof are provided by the present invention.

OBJECTS OF THE INVENTION

It is an object of the present invention to provide methods of modulating the proliferation of smooth muscle cells.

Another object of the present invention is to provide methods of inhibiting intravascular blockages.

It is a further object of the present invention to provide compositions useful for the modulation of smooth muscle cells.

Yet another object of the present invention is to provide compositions useful for the inhibition of intravascular blockages.

Still another object of the invention is to provide kits useful for the modulation of smooth muscle cells.

It is a still further object of the present invention to provide kits useful for the inhibition of intravascular blockages.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the use of a catheter in accordance with the invention to transform *in vivo* cells present on the wall of a patient's blood vessel.

SUMMARY OF THE INVENTION

The present invention is directed to methods of modulating smooth muscle cell proliferation comprising transforming smooth muscle cells with genes coding for one or more interferon polypeptides. In accordance with other embodiments of the present invention methods of inhibiting intravascular blockages are provided in which genes coding for one or more interferon polypeptides are administered to an area of vessel wall injury in an amount effective to inhibit the formation of thrombi. In further embodiments of the present invention, kits for modulating smooth muscle cell proliferation are provided comprising an intravascular device and genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier. In addition, kits for inhibiting intravascular blockages comprising an intravascular

device and genes coding for ne or more interferon polypeptides in a pharmaceutically acceptable carrier are provided. Pharmaceutical compositions comprising genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier are also provided by the present invention.

DETAILED DESCRIPTION OF THE INVENTION

Methods of modulating smooth muscle cell proliferation are provided by the present invention. The methods comprise transforming smooth muscle cells with genes coding for one or more interferon (IFN) polypeptides. In other embodiments of the present invention, an effective amount of genes coding for one or more interfereon polypeptides are administered to an area of vessel wall injury in order to inhibit thrombi formation.

IFNs are highly potent polypeptides that exert specific antiproliferative effects on various cell types, and inhibit collagen synthesis. Heyns et al., *A. Exp. Cell. Res.* 161: 297-306 (1985); Pestka et al., *Ann. Rev. Biochem.* 56: 727-77 (1987). There are three major classes of interferons, leukocyte or α IFN (IFN-α), fibroblast or beta IFN (IFN-β), and immune or gamma IFN (IFN-γ). There are at least 24 nonallelic genes or pseudogenes coding for structurally different human IFN-α which are not fully characterized. The apparent molecular weight of IFN-α subclasses ranges from 16,000 to 27,000. Their amino acid compositions are very similar and rich in leucine and glutamic acid/glutamine. They are polypeptides of 165 or 166 amino acids and their amino acid sequences are quite homologous with at least 70% sequence identity between the individual molecules (Nagata et al., *Nature* 287: 401-408 (1980); Allen et al., *Nature* 287:408-411 (1980); Zoon et al., *Interferon Res.* 2:253-60 (1982)).

IFN-β1 has a molecular weight of approximately 20,000. Its amino acid composition is similar to that observed for human- and murine-IFN-α as well as murine- and bovine-IFN-β (Langer and Pestka, *Pharmacol. Ther.* 27: 371-401 (1985); Z on et al., *Handbook Exp. Pharmacol.* 71: 79-100 (1984)).

Human-IFN-β1 is a glycoprotein which contains three cysteines forming one essential disulfide bond between amino acids 31 and 141. It is believed that the major portion of the carbohydrate moiety is linked by N-glycosylation at position 80. The functional unit of human-IFN-β appears to be a dimer.

IFN-β2, which is coinduced with IFN-β1, appears to be structurally distinct from Hu-IFN-β1 (Billiau, A., *The interferon-interleukin 1 connection. In: Interferon 9* (Academic, N.Y., N.Y. pp. 92-113, Gresser I, ed. 1987). IFN-β2 is identical to B-cell differentiation factor and mediates some biological effects which are distinct from the typical IFN effects. It has also been named interleukin-6.

IFN-γ (type II IFN) is clearly distinct from the type I (IFN-α and IFN-β1) IFNs in its physicochemical properties, size, genomic structure, chromosomal localization, and protein sequence (Vilcek, J., *Mediators of cell growth and differentiation*, Raven Press, N.Y., N.Y. pp. 92-113, 1987). The singular IFN-γ gene seems to have evolved independently. It contains introns and is localized on human chromosome 12. Upon cleavage of a 20-amino-acid signal peptide, the mature protein, which consists of 146 amino acids, is N-glycosylated at two sites, and forms dimers and tetramers constituting the biologically active forms. Although it contains two cysteines, no disulfide bond has been detected in the active molecule.

IFNs can be secreted or expressed in many mammalian types in response to various inducers such as fibroblasts, macrophages, T-lymphocytes, hematopoietic cells, and others and exhibit little cell specificity.

The IFNs exhibit a number of biological effects. In general, all phases of the cell cycle appear to be prolonged. IFNs lower the probability for cells in G0 phase to enter into the cell cycle. The G1 phase is also prolonged in actively proliferating cells following IFN treatment. It has also been observed that the time required for cells to carry out DNA replication (i.e., length of S phase) can be increased, and some cell types accumulate in S phase, failing to complete DNA synthesis after IFN treatment. A delay in progression through

G2 phase has also been noted in some cases. Sokawa et al., *Nature* 268: 236-238 (1977); Mallucci et al., *Interferon Res.* 3: 181-189 (1983); Lunblad et al., *E. Int. J. Cancer* 27: 749-754 (1981); Balkwill et al., *J. Nature (London)* 274: 798-800 (1978). In addition, IFNs can antagonize cellular responses to positive effectors of cell proliferation. The stimulation of the quiescent cells with the growth medium has been inhibited by IFN. Furthermore, the effects of the individual growth factors have also been reduced. (Sokawa et al. *supra.*; Balkwill et al. *supra;* Lundgren et al., *O.J. Gen. Virol.* 42: 589-595 (1979); Lin et al., *Biochem. Biophys. Res. Commun.* 96: 168-174 (1980)). It is believed that IFNs exert their effects through specific cell surface receptors. IFN-α and IFN-β have a common receptor which is distinct from the IFN-γ receptor. After binding, IFNs are rapidly internalized via receptor-mediated endocytosis.

Genes coding for these interferon polypeptides are employed in methods of the present invention. Gene, when used herein, is intended to connote the common meaning of the word, i.e., a DNA or RNA sequence which encodes a functional protein or RNA molecule. Genes of the present invention may be synthetic or naturally occurring.

In some aspects of the present invention, intravascular blockages are inhibited by administration of said genes. In the context of the present invention, the term "inhibition" means a partial or total inhibition of thrombi formation sufficient to lessen the severity of the vascular disorder.

The present invention is also directed to proliferation associated with any vascular and peripheral stenosis including coronary restenosis which involves intense smooth muscle cell (SMC) proliferation in response to vessel wall injury. SMCs have been demonstrated to express autocrine substances which influence their own function, such as growth and contractility. While not intending to be bound to any particular theory, it is believed that by transfecting SMCs with genes coding for one or more interferon polypeptides, SMCs

will produce IFN locally and the resulting "autocrine-paracrine" syst m in SMC with IFN gene transfection will serve as a counterbalance to growth factors and act as a downregulator of SMC proliferation.

SMCs may be transformed by genes coding for interferon polypeptides by methods known to those skilled in the art. Tranformation is the process by which cells have incorporated an exogenous gene by direct infection, transfection or other means of uptake. In preferred embodiments of the present invention, transformation is accomplished by means of a liposome-mediated transfection as described in Ausubel et al., *Current Protocols in Molecular Biology* (1991) incorporated by reference herein in its entirety. A gene coding for an interferon polypeptide is incorporated into a suitable vector such as pSG5 (Stratagene Cloning Systems, La Jolla, CA). Other vectors having characteristics useful in the present invention will b apparent to those skilled in the art. The term "vector" is well understood in the art and is synonymous with the phrase "cloning vehicle".

A solution containing one or more interferon genes incorporated in a vector is delivered into the vascular system of a patient by means of an intravascular device. An effective amount of genes coding for interferon polypeptides may eliminate all smooth muscle cell proliferation and thrombi typically associated with vessel wall injury, or may lessen the severity of stenosis in comparison with untreated vessels. Th effective amount of genes coding for interferon polypeptides will vary depending upon conditions such as, for example, patient age, weight and general state of health. One skilled in the art will be able to determine to effective amount of said genes in any particular circumstance. In some embodiments of the present invention, the genes coding for interferon polypeptides may be admininstered in conjunction with other th rapeutics found effective to limit or eliminate stenosis, such as, for xample, anti-platelet, anti-coagulation, anti-inflammatory, and vasodilation therapeutics. The solution may

also contain a proteolytic enzyme such as disparse, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, lipases may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Any blood vessel may be treated in accordance with this invention. For example, arteries, veins and capillaries are encompassed within the scope of the present invention. The vessel surface can be prepared by mechanical denudation alone, in combination with small amounts of proteolytic enzymes such as dispase, trypsin, collagenase or cathepsin. The denudation conditions are adjusted to achieve the appropriate loss of cells from the vessel wall.

Intravascular devices known to those skilled in the art are encompassed by the present invention. For example, injection and transcatheter delivery devices are useful in the present invention. In some preferred embodiments of the present invention a catheter device may be employed. Any catheter means which can be held firmly in place within the blood vessel with little or no resulting mechanical injury and which can deliver a solution containing said gene is encompassed by the present invention. In one preferred embodiment of the present invention, a catheter as illustrated in Figure 1 is provided. In Figure 1 5 is the wall of the blood vessel. The figure shows the catheter body 4 held in place by the inflation of inflatable balloon means 2, equipped with a plurality of apertures 3. Said apertures may range in diameter from about 10μm to about 50μm. More preferably the diameter of the apertures is from about 15 μm to about 40μm. Most preferably said apertures range in diameter from about 20μm to about 30μm. While Figure 1 illustrate three apertures, the figure is not intended to limit the number of apertures in said balloon means. The number of apertures will be a function of the aperture diameter and pressure exerted necessary for optimal delivery of the perfusate to the smooth muscle cells, the determination of which is within the ambit of one skilled in the art. Of course the balloon means will have at least one

aperture. Preferably the balloon means has from about 1 to about 50 apertures. From about 5 to about 25 apertures is still more preferable. Most preferably, the balloon means has from about 10 to about 15 apertures. By exerting pressure on the solution (perfusate), the balloon inflates and perfusion fluid sprays from the aperture of the balloon, thereby impregnating the perfusate within the wall of the blood vessel. At least about 2 atmospheres (atm) of pressure is exerted on the perfusate. Preferably at least from about 2 to about 10 atm of pressure is exerted on the perfusate. Most preferably from about 2 to about 5 atm pressure is exerted on the perfusate. This procedure involves occlusion of the blood vessel which can, after prolonged occlusion, lead to myocardial infarction. Therefore occlusion of the blood vessel should be of limited duration. Occlusion should last preferably no longer than about one to two minutes minute.

Another embodiment of the present invention provides kits for modulating smooth muscle cell proliferation containing an intravascular device and genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier. Kits for inhibiting intravascular blockages comprising an intravascular device and genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier are also encompassed by the present invention. In some preferred embodiments of the present invention, the intravascular device is a catheter. The pharmaceutically acceptable carrier may be a solution containing a proteolytic enzyme such as disparse, trypsin, collagenase, papain, pepsin, or chymotrypsin. In addition to proteolytic enzymes, lipases may be used. As a mild detergent, the solution may contain NP-40, Triton X100, deoxycholate, SDS or the like.

Pharmaceutical compositions comprising genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier are also provided by the present invention.

EXAMPLES

Other features of the present invention will become apparent in the course of the following Examples. These Examples are illustrative, but are not meant to be limiting of the present invention.

EXAMPLE 1 Selection of the most potent IFN-inhibitor of SMC proliferation

A. SMC culture

In vitro studies will be carried out in human (hSMC) and porcine (pSMC) SMC cultures. The IFN(s) exhibiting the strongest inhibition of SMC growth will be chosen for the gene transfection study. The human recombinant IFNs (hr-IFN) will be tested first in hSMC in different doses for 3 days' exposure. The doses of IFNs and the duration of exposure will be modified depending on the initial results. In addition, should the inhibition of SMC growth be less than expected, a combination of two IFNs (e.g., IFN-α or IFN-β+IFN-γ) will be tested to augment their antiproliferative effects. The same procedure will be applied in pSMC. If hr-IFN shows a suboptimal effect in pSMC, mouse recombinant IFN (mr-IFN) will be tested.

Cells will be cultured at 37°C in a humidified, 5% CO_2/95% air atmosphere. Triplicate plates will be analyzed for each experimental growth condition. Passages 3 to 10 will be used in all experiments. The cell type will be identified as SMC by the presence of "hill and valley" appearance, and SMC specific myosin will also be determined by indirect immunofluorescence.

1. Human SMC culture

In accordance with methods set forth in Tan et al., *Biochem. Biophy. Res. Commun.* 163: 84-92 (1989) adult human iliac arteries are obtained from brain-dead, heart-beating cadaver renal donors. After harvesting the endothelial cells by collagenase treatment, the adventitia will be removed and the medal tissue will be minced. Th minces will be placed in gelatin-coated flasks and incubated in complete medium (CM) including M 199 medium, 2 mM glutamine, 10% fetal bovine serum

(FBS). Endothelial cell growth factor (ECGF, 75 μg/ml) and heparin (100 μg/ml) are added only to the medium of primary cell cultures. The explants are changed with fresh CM twice a week and harvested at confluency. Thereafter, the cells are harvested weekly with 0.25% trypsin, and are seeded at 10^4 cells/cm^2 in the same CM.

2. Porcine SMC culture

pSMC will be isolated from porcine aorta according to the explant method of Ross, R., *J. Cell Biol.* 50: 172-186 (1989). The aorta is removed and fascia cleaned. The medial layer of aorta is cut into 1 mm^2 pieces and placed in 100 mm culture dishes containing DMEM, 10% FBS, 2mM glutamine. ECGF (75 μg/ml) and heparin (60 μg/ml) will be added to the primary cell culture medium. The medium is replaced at 3-day intervals. After 2 weeks of cultivation, outgrowth of cells is observed. Serial subcultures are prepared using splitting ratios of 1:3 or 1:4. In all experiments cells from 3-10 subculture will be used.

B. Growth inhibition experiments

Cells will be plated in 16 mm multiwell plates at densities of 10^4 cells per well in normal growth medium. After 24 hours, their growth will be arrested by reducing FBS to 0.4%. In this condition approximately 90% of cells are arrested in $G_0(G_1)$. Cells are then divided into the following groups:

Control group: Cells will be incubated with CM for 72 hours;

Treated group: Cells will be incubated with CM and respective IFNs in 5 different doses for 72 hours. The following IFNs will be tested:

• hr IFN-α (Schering Corporation, Kenilworth, NJ) is expressed in *E. coli*. Specific activity is >$3x10^8$ lU/mg. The purity is homogeneous on HPLC and SDS PAGE.

• hr IFN-β1 (Berlex Laboratories, Inc. Alameda, CA) is separated from BHK cells. Specific activity is >$2x10^8$ IU/mg and purity is >95%.

• hr IFN-β2 (Boehringer Manheim Co., Indianapolis, IN) is expressed in *E. coli*. Specific activity is >$2x10^8$ lU/mg.

Purity is >98% (SDS-PAGE).

• hr IFN-γ (Boehringer Mannheim Co., Indianapolis, IN) is expressed in *E. coli*. Specific activity is >$2x10^7$ lU/mg (EMC virus/WISH cell system). Purity is >99% (HPLC).

C. Cell inhibition assay

After cells are trypsinized, they are counted in duplicated samples using a Coulter counter (Coulter Electronics, Inc. Hialeah, FL). Trypsinized cultures will be routinely checked by a direct microscopic examination to ascertain that the trypsinization procedure does not lyse the cells (i.e., presence of cell debris) and to ensure that all cells are removed from the wells. The net growth of SMC in the control and IFNs-treated cultures is obtained by subtracting the starting cell number (at the time the cells are released from G0) from the cell number at the end of the experiment in the control and IFNs-treated cultures, respectively. The degree of inhibition is determined from the following formula:

$$\% \text{ inhibition} = \left(1 - \frac{\text{net growth in IFN-treated}}{\text{net growth in controls}}\right) \times 100$$

D. Collagen synthesis assay

A collagen synthesis assay is performed in accordance with methods provided in Tan et al., *Biochem. Biophy. Res. Commun.* 163: 84-92 (1989). After effective IFN is chosen, the collagen synthesis will be evaluated using optimal dose and exposure time. Confluent cultures of hSMC grown with or without IFN(s) are preincubated with 40 μg/ml of ascorbic acid 2 hours prior to the addition of 20 μCi/ml of L-[2,3,4,5-^{3}H] proline (108.6 Ci/mmol; New England Nuclear, Boston, MA). After 16 hours, the incubation is terminated by separating the medium from the cells and cooling the fractions to 4°C. Various protease inhibitors are added to the medium samples. The cells are rinsed with PBS, extracted with 0.4 M NaCl-Tris buffer (pH 7.5) containing protease inhibitors, and sonicated at 60 Hz for 30 seconds. To quantitate the synthesis of [^{3}H] hydroxyproline, aliquots of medium and homogenized cells are

dialyzed against tap water, hydrolyzed in 6 N HCl in sealed tubes at 115°C for 24 hours and assayed for nondialyzable [^{3}H] hydroxyproline using a specific radiochemical method. Tan et al., *Biochem. Biophy. Res. Commun.* 163: 84-92 (1989). The values are normalized for cellular protein and DNA.

EXAMPLE 2 Construction of expression vectors containing IFN gene

A. Design of the vector structure

pSG5 (Stratagene Cloning Systems, La Jolla, CA) is a 4.1 kB eukaryotic expression vector constructed by combining pKCR2 and Stratagene's pBS vector. The vector contains the early SV40 promoter to facilitate *in vivo* expression and the T7 bacteriophage promoter to facilitate *in vitro* transcription of cloned inserts. Intron II of the rabbit b-globin gene facilitates splicing of expressed transcript, and a polyadenylation signal increases the level of expression. Three unique restriction sites EcoR 1, BamH1 and Bgl II are located downstream from the promoter sites. The pSG5 vector has been used for *in vivo* and *in vitro* expression of a wide variety of cells. The cDNA encoding the designed protein-IFN (R & D SYSTEMS, Minneapolis, MN) is synthesized using standard phosphoramidite chemistry and the oligonucleotides, in the laboratories of British Bio-technology Limited (BBL) and is suitable for expression in eukaryotic system. The final protein sequence is identical to the natural sequence. IFN DNA will be inserted into the pSG5 at multiple cloning sites by restriction enzyme digestion and DNA ligation (Sambrook et al., ed., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY 1989). The plasmid pSG5 containing IFN DNA will b grown in *E. coli*, and the recombinant DNA- pSG5/IFN will be purified by a miniprep procedure or by CsCl/ethidium bromide centrifugation. (*Current Protocols in Molecular Biology*, Green Publishing Association, 1991).

B. Evaluation f th transient expression of IFN

COS cells are African gr en monkey kidney cells that have been transformed with an origin-defective SV40 virus,

which has integrated into COS cell chromosomal DNA, and can be used to produce high level and short-term expression of designed protein. If transfected DNA encodes a secreted protein, up to 10 μg of protein can be recovered from supernatant of the transfected COS cells one week posttransfection.

COS-7 (ATCC) cells will be seeded at 2 x 10^6 and grow overnight to 50% confluent. pSG5/IFN will be transfected to COS cells using optimal condition of liposome mediated transfection procedure. Felgner et al., *Biochem* 84: 7413-7417 (1987). 72 hours after transfection, the supernatant will be harvested and secret protein will be detected by Western blotting (Sambrook et al., *supra*).

C. Analysis of biologically active protein from SMC

The supernatant harvested from transfected COS cells will be added to SMC. The cell growth inhibition will be monitored. Inhibition of SMC growth is observed.

EXAMPLE 3 Feasibility and efficiency of gene transfection in SMC culture

Recently, the feasibility of direct gene transfer into the vascular segment by retroviral infection and liposome-mediated transfection has been demonstrated. The marker gene product, β-galactosidase (β-gal) or luciferase, could be detected in a variety of cell types in the vessel wall from 3 days to 5 months (Nabel et al., *Science* 249: 1285-1288 (1990); Chang et al., *Circulation* 83: 2007-2011 (1991)). Taking into account the safety concern related to retroviral-mediated gene transfer in patients, we intend to use liposome-mediated gene transfer method. Felgner et al., *Biochem.* 84: 7413-7417 (1987).

Three parameters such as the concentrations of lipid and DNA, as well as the incubation time of the liposome-DNA complex, are important in gene transfection by cationic liposomes. Therefore, we will determine the optimal condition for liposom -mediated g ne transfer in SMC. These three factors will be systematically examined according to standard

procedures (Ausubel et al., ed. *Current Protocols in Molecular Biology* 1991) to obtain optimal transfection. After the optimal condition is selected, this procedure will be used in all gene transfection experiments.

The pSV-β-galactosidase vector (Promega, Madison, WI) is designed as a positive control vector for the monitoring of transfection efficiencies of mammalian cells. The SV40 early promoter and enhancer drive transcription of the bacterial *lac Z* gene which, in turn, is translated into the β-gal. It has been reported as an excellent reporter enzyme which can be quickly and directly assayed in cell extracts and stained *in situ*.

The plasmid pSV-β-gal (mammalian expression vector) will be grown in *Escherichia coli (E. coli)*, and purified. Sambrook et al., ed., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY 1989. Purity will be confirmed by 8% agarose gel electrophoresis, demonstrating the absence of bacterial cellular DNA. The purified pSV-β-gal will be mixed with N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) and dioleoyl phosphatidylethanolamine (DOPE) (Bethesda Research Laboratories, Gaithersburg, MD). The lipid-DNA complexes are immediately formed due to ionic interactions between the positively charged group on the DOTMA molecule and negatively charged phosphate groups on the DNA. SMC will be plated in 6-well tissue culture dishes at 5×10^5 cells/well, and grow overnight to 80% confluency. The cells are then washed with serum free medium and the lipid-DNA mixture, made according to standard procedure provided in Sambrook et al., ed., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, NY 1989, is added. The cells will be incubated for 6 hours at 37°C. CM is then added to the cells and cells are incubated for an additional 16 hours. The medium is replaced with fresh CM, and the incubation is continued for the next 48 hours.

Forty eight hours after transfection, cells are washed with PBS buffer and cell extracts will be prepared by freeze-thaw methods. The cell extracts will be added with

phosphate buffer and the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside). After incubation at 37°C, the reaction will be terminated by addition of Na_2CO_3, and the absorbance is read in a spectrophotometer at 420nm. The optimal condition will be the one which shows the highest reading of absorbance.

EXAMPLE 4 Evaluation of IFN gene expression and its antiproliferative effect in SMC culture

The inducibility and efficiency of the IFN gene transfection will be evaluated by measuring IFN protein or IFN RNA. Furthermore, the function of the IFN gene will be assessed by measuring the recipient cell growth.

A. IFN protein measurement by Western blotting

pSG5-IFN will be transfected into SMC under optimal condition as described in Example 3. 72 hours post-transfection, cells will be washed and lysed, and the cell lysates will be solubilized with sodium dodecyl sulfate (SDS). After separation by polyacrylamide gel electrophoresis, the antigens will be electrophoretically transferred to nitrocellulose paper. The paper is then blocked to prevent nonspecific binding of antibody and probed with IFN antibody (R & D Systems, Minneapolis, MN). The antibody will be detected by a horseradish peroxidase (HRPO)-antiimmunoglobulin conjugate and visualized by incubating the filter paper in the presence of a precipitable substrate.

B. *In situ* hybridization of cellular RNA for IFN

In situ hybridization of cellular RNA will be necessary only if IFN protein is not detected. This will help to define whether the negative result is related to insufficient quantity of IFN protein, or the failure of IFN gene expression. In accordance with methods of Ausubel et al., *Current Protocols in Molecular Biology* 14.0.1-14.6.8 (1991) cells are suspended in a serum-free medium (2×10^7 cells/ml density) and fixed to poly-L-lysine-coated slides with 4% paraformaldehyde. The slides are dehydrated through a series of 5-minute incubations in 50%, 70%, 95% and 100% ethanol. After drying, slide boxes containing desiccant will be stored

at -70°C. Specimens will be dewaxed, rehydrated and denatured to remove some of the proteins and make the RNA transcripts more accessible for hybridization. RNA contained in the specimens is hybridized to a [^{35}S]UTP-labeled riboprobe for 4 hours. Specimens are then washed and digested with RNase to remove excess and nonspecifically hybridized probe. IFN RNA will be detected using film autoradiography.

C. Measurement of transfected cell growth

The transfected cell growth will be evaluated by counting and comparing the number of pSG5-IFN or pSG5 transfected cells at 3 and 5 days posttransfection.

Percent inhibition of transfected cells will be calculated using the formula described in Example 1. It is believed that cell growth will be inhibited with detectable expression of IFN mRNA or synthesis of IFN protein.

EXAMPLE 5 Feasibility and stability of *in vivo* gene transfection

Based on the results of the above *in vitro* series of experiments, the most potent IFN gene will be selected for *in vivo* gene transfection.

A. Gene delivery system

There are two available catheter systems designed to deliver pharmacologic agents into the vascular wall. The first consists of two inflatable balloons occluding a segment of the vessel, and a central instillation port. Using this system or its modification, successful transfer of reporter genes into the arterial wall has been demonstrated after prolonged exposure of the vessel wall to the transfection solution. Lin et al., *Circulation* 82: 2217-2221 (1990). Because of the possibility of myocardial infarction after prolonged coronary artery occlusion, this system is not practical for its application in the coronary circulation. The second devic consists of a perforated (i.e., porous) balloon. The balloon inflates when pressure is exerted on the perfusate. Wolinsky et al., *J. Am. Coll. Cardiol.* 15: 475-481 (1990). Then depending on the exerted pressure, the perfusion fluid sprays

from the multiple holes, 25 μm in diameter, and impregnates different layers of the vessel wall. This device which allows for deeper penetration of the perfusate within the wall, and most importantly inflation for only one minute, resulted in the staining of the entire media. Wolinksy et al., *supra*. Since coronary occlusion for one minute is commonly used and well tolerated during coronary angioplasty in patients, a perforated balloon will be used for the transfection studies *in vivo*.

Using anesthetized porcine model, standard nonperforated balloon catheter will be advanced to the surgically exposed femoral artery. Then, the studied segment will be denuded by the inflation of the balloon. The side branches will be occluded and the segment of the artery will be marked. Next, the perforated balloon will be advanced to the denuded portion of the femoral artery. Suspension of colored (nonradioactive) microspheres (5 μm in diameter) will be injected through a porous balloon into the vessel wall. The direct contact between a perforated balloon and the vessel wall will be achieved by exerting pressures of 2 atm or more on the perfusate. Each inflation will last one minute, and inflation pressures of 2, 3, 4, and 5 atmospheres (atm) will be tested. Different colors of microspheres will be suspended in the perfusate for each inflation pressure tested. Special attention will be paid to remove the remaining microspheres before the next set of microspheres will be instilled. Five arterial segments will be studied for each inflation pressure. The animals will be sacrificed with an overdose of pentobarbital and perfused antegradely with 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate to allow fixation of the arteries *in situ*. Paraffin-embedded rings will be cut into 7 μm sections and stained with hematoxylin eosin, trichrome, and elastic tissue stains. Using the light microscopy, the penetration of microspheres in the vascular wall will be assessed. The inflation pressure resulting in penetration of the microspheres through the entire media will be chosen.

B. The expression of a report r gene *in vivo*

Direct gene transfer of a B-galactosidase expression vector into the arterial wall will be studied in nonatherosclerotic porcine model. Farm pigs will be anesthetized with pentobarbital (20mg/kg), intubated and mechanically ventilated. Bilateral femoral arteries will be denuded using a standard balloon catheter. The lipid-DNA mixture will be prepared as described in Example 3. A perforated balloon will be advanced to the denuded segment of the femoral artery under the fluoroscopic guidance. Using the perfusion pressure, resulting in the penetration of the media by the perfusate as described in Example 4, the transfection solution will be delivered into the vascular wall. The lipid solution not containing DNA will be delivered to the contralateral artery. Afterwards, catheters will be removed, arteries repaired, and the animals will be allowed to recover. The animals will be sacrificed at 24 hours, 1, 3, and 6 months after the gene transfer. The vessel segments are quickly frozen in semisolid isopentane, cooled in liquid nitrogen, and sectioned at 6 μm in a cryostat. The cryosections are fixed in 1.25% glutaraldehyde for 15 minutes at 4°C and stained with X-gal chromagen at 37°C for 18 hours. The specimens are then counterstained with Harris' hematoxylin and mounted in glycerin-gelatin. After cleared in ascending alcohols and xylene, they are mounted in permanent mounting medium. β-galactosidase activity will be indicated by visible areas of blue coloration under microscope. The intensity of arterial staining will be judged by a visual inspection from + to +++. Control sections will be run without the b-galactoside substrate to eliminate the false-positive staining.

EXAMPLE 6 Effect of IFN gene transfection in preventing restenosis

A. Liposome-mediated gene transfer into th c r nary artery

The atherosclerotic porcine model of coronary artery denudation will be used in these experiments. This model

offers several advantages such as comparable size of coronary arteries with human coronary circulation, and similar platelet characteristics as well as histology of stenotic lesions.

Yucatan miniswine, weighing about 20-30 kg, will be placed on an atherogenic diet consisting of 2% cholesterol, 15% fat, and 1.5% sodium cholate. One month after the initiation of the diet, Yucatan miniswine will be sedated with intramuscular injection of ketamine hydrochloride (20-30 mg/kg), 0.22 mg/kg acepromazine and 0.05 mg/kg atropine sulfate. After intubation, 0.5% halothane mixture with room air will be given to maintain anesthesia. Using sterile technique, the right femoral artery will be isolated. An 8F hemostatic sheath will be inserted and 200 U/kg intravenous heparin will be given. Nifedipine (10 mg) buccally will be administered to prevent coronary artery spasm. In addition, bretylium tosylate (5 mg/kg iv) will be given every 15 minutes to prevent ventricular fibrillation. The left main coronary artery will be entered using an 8F large lumen guiding catheter. Intracoronary nitroglycerin (200 ucg) will be given. The left anterior descending coronary artery will be dilated first with a 2.5 or 3.0 mm standard balloon catheter in order to achieve endothelial denudation and medial injury. Then a perforated balloon will be advanced to denuded segment of the left anterior descending coronary artery. The animals will be randomized to receive either lipofection solution (control group, n=10) or the lipid-pSG5-IFN complex (treated group, n=10). The perfusate will be delivered over a one minute period, as described in Example 4. Following an additional dose of intracoronary nitroglycerin, coronary angiogram will be recorded. Then, all catheters will be removed, and the wound will be closed surgically. One inch of 2% nitroglycerin ointment will be applied topically to the shaved area at the end of the procedure. The animals will be allowed to recover, and adjunctive therapy will include: aspirin (1 mg/kg/day po) and ampicillin 250 mg intramuscularly for the next two days. The animals will be maintained on an atherogenic diet for 3 months following surgery.

B. Evaluation of antipr lif rative effect

Three months after coronary dilatation, using the same preanesthetic, anesthetic, and antispasm protocols as described in Example 5, follow-up coronary angiography will be performed. Special care will be taken to obtain angiographic views in the same projections as those obtained after coronary dilatation. The animals will then be sacrificed. Using pressure fixation technique, arterial segments will be preserved for future histology.

1. Angiographic patency and loss of the luminal diameter

Quantitative analysis of coronary angiograms immediately after the stent implantation, and on follow-up angiography 3 months later, will be performed using a computer based coronary angiography system (ADAC). Appropriate cine frames will be digitally acquired by the imaging computer via a television camera converter mounted on a cine 35 mm viewer (General Electric, CAPS 35 projector). The video signal is then digitized at 512 X 512 X 8 bit resolution onto a digital angiographic computer (ADAC Laboratories, Model DPS-41 00). Images are then magnified 4 fold using bilinear interpolation. After the region of interest is identified by the operator, an automatic edge detection algorithm is then used to determine the arterial contour by assessing brightness along scan lines perpendicular to the centerline. Quantitative measurements are determined using the coronary catheter as a scaling device. Using this automatic edge detection system, 3 quantitative parameters of stenosis severity will be measured: minimal luminal diameter, percent diameter stenosis, percent area stenosis.

2. Microscopic studies

Segments of the left anterior descending coronary artery from the control and treated animals will be fixed in 4% formaldehyde in 0.1 M phosphate buffer for one hour at 4°C. After dehydration in graded ethanols, they will be embedded in paraffin. Five μm sections will be cut and the sections stained with hematoxylin eosin, tichrome and elastic tissue

stains. Sections fr m the control and treated s gments will be analyzed for the presence of thrombus and neointimal proliferation. In addition, sections will be stained with antibodies directed against von Wildebrand factor, and against a-actin. Cells reacting with antibodies against von Wildebrand factor are endothelial cells, whereas cells reacting with antibodies against a-actin represent smooth muscle cells. The antibody staining will be performed according to previously described methodology (Tsukada et al., *AJP* 126: 51-6 (1987)).

3. Mortality

Mortality between 24 hours and 3 months after coronary dilatation will be a major endpoint of the study. It is anticipated that mortality beyond the time of surgical recovery (i.e., after the first 24 hours) is most likely related to the subacute thrombosis of the dilated segment of the coronary artery.

EXAMPLE 7 Evaluation of IFN gene expression in the coronary artery

This analysis will determine the relationship between efficiency of gene transfection and antiproliferative effect. In additicn, it may also provide important information related to quantities of gene transfection.

The animals are killed 4, 8 and 12 weeks after IFN gene transfection, and the transfected artery sections are excised and prepared for *in situ* hybridization.

A. IFN protein in coronary artery measured by immunohistochemical assay

The tissue samples are quick-frozen in liquid N_2, immersed in cold Cryokwik and then sectioned. The cryosections are collected onto poly-L-lysin coated slide and fixed with paraformaldehyde. After dehydrated, slides are incubated with IFN antibody and flurochrome conjugate. The positive area of the tissue specimen will be detected under microscope and photographed.

B. *In situ* hybridization of vascular IFN mRNA

In situ hybridization of vascular mRNA is necessary only if IFN protein is undetectable.

The cryosections will be prepared as described in Example 7. The cryosections on poly-L-lysine-coated slides are treated with pronase and acetylated. IFN DNA probe will be labeled with [^{35}S] and incubated with specimens. After washing, slides will be dried and hybridized probe will be detected by autoradiography.

CLAIMS

What is claimed is:

1. A method of modulating smooth muscle cell proliferation comprising transforming smooth muscle cells with genes coding for one or more interferon polypeptides.

2. The method of claim 1 wherein said smooth muscle cells are located in the wall of a blood vessel.

3. The method of claim 1 wherein said interferon is one of the group consisting of alpha, $beta_1$, $beta_2$ and gamma interferon.

4. A method of inhibiting intravascular blockages comprising admininstering genes coding for one or more interferon polypeptides to an area of vessel wall injury in an amount effective to inhibit the formation of thrombi.

5. The method of claim 4 wherein said interferon is one of the group consisting of alpha, $beta_1$, $beta_2$ and gamma interferon.

6. A kit for modulating smooth muscle cell proliferation comprising an intravascular device and genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier.

7. A kit for inhibiting intravascular blockages comprising an intravascular device and genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier.

8. A pharmaceutical composition comprising genes coding for one or more interferon polypeptides in a pharmaceutically acceptable carrier.

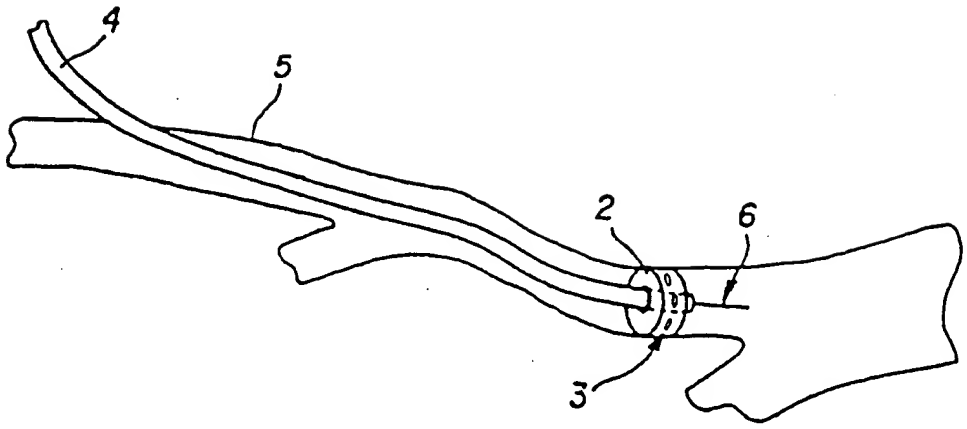

FIG. 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01072

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01N 43/04; A61K 31/70, 37/66
US CL :514/44; 424/85.4, 85.5, 85.6, 85.7

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 424/85.4, 85.5, 85.6, 85.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and Chemical Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,675,184 (Hasegawa et al) 23 June 1987, abstract and col. 2, lines 10-48.	8
Y	Science, Volume 249, issued 14 September 1990, E.G. Nabel et al, "Site-Specific Gene Expression in Vivo by Direct Gene Transfer into the Arterial Wall", pages 1285-1288, especially page 1286, figs. 1 and 2 and abstract.	1-7
Y	Circulation Research, Volume 63, Number 4, issued October 1988, G.K. Hansson et al, "Gamma-Interferon Regulates Vascular Smooth Muscle Proliferation and Ia Antigen Expression In Vivo and In Vitro", pages 712-719, especially page 712, parag. 4.	1-7

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

11 May 1993

Date of mailing of the international search report

17 MAY 1993

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. NOT APPLICABLE

Authorized officer

DEBORAH CROUCH, PH.D.

Telephone No. (703) 308-0196

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